were also found in humans (Fig. 1a). On the other hand, genotypes GII.12, GII.3, GII.6 and GII.14 were not identified in the environment, but only in humans. The remaining genotypes were observed infrequently and GII.6 strains were sufficiently distinct to preclude any epidemiological link (Fig. 1b).

Except for GGIV and GI.6 all genotypes identified in the environment were also detected in humans. Failure to detect GIV in humans could be explained by the fact that initial laboratory diagnosis of NoVs in humans only included primers for NoV GI and GII detection. The absence of genotypes GII.6 and GII.14 in wastewater is probably due to their lower prevalence in the population.

Interestingly, the diversity of NoV GGII strains in our study was higher than in a contemporary French study [11] based on outbreak surveillance, despite Luxembourg's much smaller population and shorter study duration. All six genotypes and GII.4 sub-lineages detected in 245 NoV GGII associated outbreaks in France were also found in our study. Furthermore, genotypes GII.c/GII.12 and GII.14 were found in the present study but not in the French one. This highlights the usefulness of sample collection in wastewater plants and sporadic cases in obtaining a more complete picture of overall NoV circulation.

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Transparency Declaration

There are no conflicts of interest to declare.

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Detection of West Nile virus lineage 2 in mosquitoes during a human outbreak in Greece

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Abstract

A human outbreak of West Nile virus (WNV) infections occurred in 2010 in central Macedonia, northern Greece. Most cases were observed close to four rivers forming a large Delta, a major Mediterranean wetland. WNV lineage 2 sequences were obtained from two pools of *Culex pipiens* mosquitoes trapped in sites where encephalitis cases occurred a few days before the trapping. The Greek strain showed the highest homology to Hungarian and South African strains, differing from the Russian WNV lineage 2 strain, which suggests that at least two lineage 2 strains have been introduced and established in Europe, causing severe disease to humans.

Keywords: Greece, lineage 2, mosquito, outbreak, West Nile virus

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West Nile virus (WNV) is a mosquito-transmitted flavivirus, which is maintained in an enzootic cycle between birds (amplifying hosts) and ornithophilic mosquitoes (vectors), mainly of the *Culex* species, while humans, horses and other mammals are incidental hosts. Although the majority of infections are either asymptomatic or mild febrile cases, approximately 1% of them present with neurological manifestations, with elderly and immunocompromized persons highly affected [1]. Since the 1990s WNV has become a major public health concern, as sporadic cases and outbreaks among humans and equines have been reported in several Mediterranean regions [2,3]. The interest in WNV increased after the large human outbreaks in Romania and the United States [4–6]. WNV infections had never been documented in Greece. However, WNV neutralizing antibodies had been detected in 2007 in 4/392 residents of an area close to the Axios Delta in northern Greece, which is a major wetland and resting site for migratory birds [7]. This area proved to be the epicentre of the large outbreak of WNV infections in central Macedonia, Greece, in summer 2010 [8]. The first 10 cases had been diagnosed on 5 August; all of them were central nervous system infections (encephalitis, meningitis and meningoencephalitis), and two of them were accompanied by exanthema. By 2 September 2010, 148 neuroinvasive cases had been laboratory diagnosed, and 15 of them were fatal; most cases

FIG. 1. Map of northern Greece showing the areas where the mosquito collection was performed. Human cases of West Nile virus (WNV) infection were observed in the same areas. Purple pins show the places (Nea Santa and Gypsochori) where WNV-positive mosquitoes were detected.

occurred in the Imathia, Kilkis, Pella, Pieria and Thessaloniki prefectures [9].

During 9–31 August 2010, 3524 adult mosquitoes were collected by using CO_2 traps in the places where the cases had been observed (Fig. 1). Traps were set as soon as possible after the laboratory diagnosis of the cases. Among the 3524 mosquitoes, 3232 were *Culex* spp., 226 *Aedes* spp. and 66 *Anopheles* spp. Mosquitoes were pooled according to the collection site, date and genus. Each pool consisted of no more than 50 mosquitoes. Viral RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) and RT-nested PCRs were performed using two sets of primers: one set was theoretically able to detect all flaviviruses, and the second set was specific for WNV [10,11]. Bands of the expected sizes (146 and 106 bp, respectively) were obtained on two pools of *Culex pipiens* mosquitoes. Mosquitoes of the first positive pool (50 mosquitoes) were trapped during the night of 10–11 August 2010 at Nea Santa, a village in the Kilkis prefecture, 27 km northeast of Thessaloniki (40°50′30″N, 22°54′52″E), while mosquitoes of the second positive pool (25 mosquitoes) were trapped during the night of 26–27 August 2010 at Gypsochori, a village in the Pella prefecture, 50 km northwest of Thessaloniki (40°48′20″N, 22°15′7″E) (Fig. 1). Encephalitis cases had been observed in these places a few days before the mosquito collection; WNV-specific IgM antibodies were detected by μ-capture ELISA (Focus Technologies, Cypress, CA, USA) in serum and cerebrospinal fluid samples taken on 9 August from the patient in Nea Santa, while samples from the patient in Gypsochori were taken on 17 August. Sequences of both mosquito pools were identical in both PCRs. Use of the BLAST tool revealed that the Greek sequences belonged to WNV lineage 2 strains, as they were identical to those derived from a dead goshawk in Hungary in 2004 and from a fatal human case in South Africa in 1989 [12,13]. A phylogenetic tree based on a 146-nt fragment of the NS5 gene is seen in Fig. 2 (similar clustering was seen using the 106-bp fragment; the phylogenetic tree is not shown). The 146-nt sequence

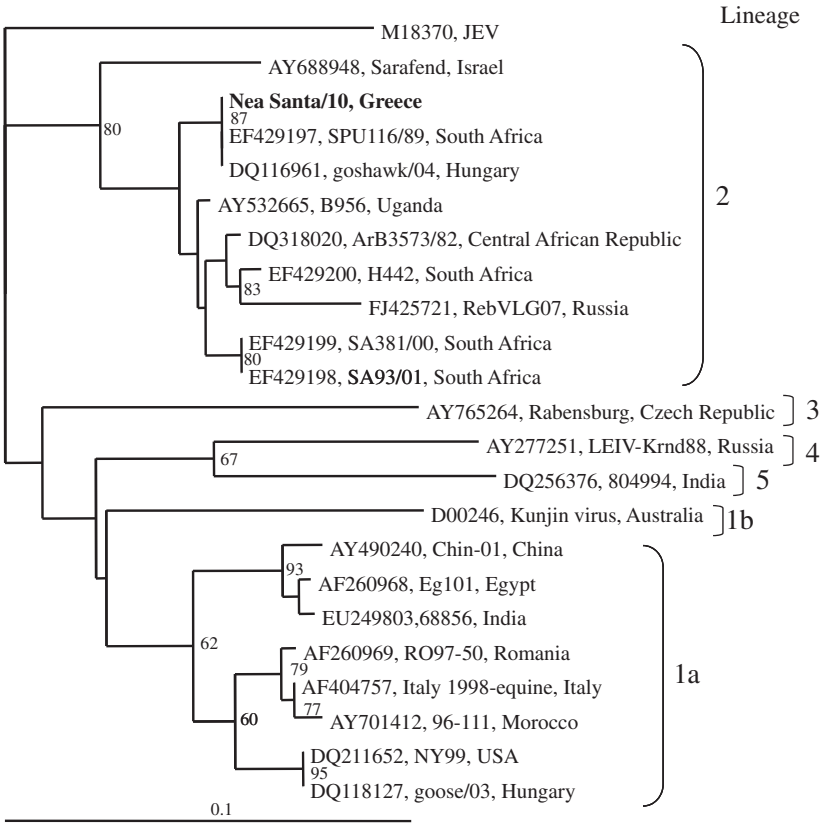

FIG. 2. Phylogenetic tree based on 146-nt fragment of the NS5 gene of WNV, constructed by using PHYLIP software with the neighbour-joining method and Kimura 2-parameter distance matrix. Japanese encephalitis virus (JEV) was used as outgroup. The numbers at the nodes indicate percentage bootstrap replicates of 100; values below 60% are not shown. Horizontal distances are proportional to the nucleotide differences. The scale bar indicates 10% nucleotide sequence divergence. Sequences in the tree are indicated as GenBank accession number, strain name, country. Strain of the present study is shown in bold.

(Nea Santa-Greece-2010) was submitted to GenBank database under the accession number HQ230576. Although the phylogenetic analysis was based on a short genome fragment, it is evident that the Russian WNV strain RebVLG07, isolated during an outbreak in Volgograd, Russia, in 2007 [14], forms a distinct subclade in the WNV lineage 2 strains, suggesting that at least two different virus introductions from Africa took place. Determination of nucleotide sequences of a larger genome fragment or, even better, of the whole genome of the Greek strain, will give better insight into the exact relation with other WNV strains, and will elucidate the probable epidemiological links, with migratory birds playing an important role.

The detection of WNV lineage 2 sequences in mosquitos collected in two different sites, exactly where WNV encephalitis cases had been diagnosed a few days before, gives strong evidence that this strain might be associated with the outbreak; the detection of WNV RNA in clinical samples will confirm the results. In addition, the possibility that a second WNV strain may be co-circulating in the region cannot be excluded. Recent outbreaks in Mediterranean countries and in North America were caused by WNV lineage 1 strains. Prior to 2004, lineage 2 strains were detected only in sub-Saharian Africa and Madagascar. Several authors addressed the pathogenicity of WNV lineage 2 strains in South Africa in humans and horses [13, 15–17]. Because only a few cases of neurological disease have been reported from S. Africa, endemic lineage 2 strains were postulated to be of low virulence; however, recent studies suggest that lineage 2 WNV may be significantly underestimated as a cause of neurological disease in S. Africa [18]. In 2004 a WNV lineage 2 strain was detected in birds in Hungary, and since then it has been detected sporadically in Hungary and the eastern part of Austria [12, 19]. Another lineage 2 strain was detected during a WNV outbreak in Russia in 2007 [14]. The genetic distance between the two lineages is relatively high (approximately 20% at nucleotide level), a fact that facilitates the virus spread in susceptible populations, especially when ecological factors are favourable.

Culex spp. is the most common species in Greece. Previous studies have shown that *C. pipiens* is the dominant and endophilic species in rural areas in central Macedonia, and it was being collected for many years by using the human bait method (Mourelatos, personal observations). Testing of additional mosquito pools is still in progress in Greece, while whole genome nucleotide determination procedures are ongoing. Results of the present study, although preliminary, suggest that WNV lineage 2 strains were imported into and established in Europe and might be a great public health threat for Europe or other continents.

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Transparency Declaration

The authors declare no conflicts of interest.

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Prevalence of antibodies to phleboviruses and flaviviruses in Peja, Kosovo

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Abstract

In order to investigate the current and past activity of phlebovirus and flavivirus in Kosovo, a seroprevalence study among 200 blood donors was performed. Positive results were obtained for the phleboviruses TOSV and SFNV, and for a flavivirus of the Japanese Encephalitis group. No positive results for TBEV were observed.

Keywords: Arbovirus, flavivirus, Kosovo, phlebovirus, seroprevalence

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The last decades have been characterized by the emergence in the human population of new infectious agents from animal reservoirs, but also by the epidemic resurgence of well-known arboviral diseases, such as dengue, West Nile and chikungunya fever [1,2].

Other arboviruses, such as the Toscana virus (TOSV) and the tick-borne encephalitis virus (TBEV), are known to be endemic in some European countries. TOSV is mainly found in the Mediterranean basin, whereas TBEV, which is commonly found in central Europe, is expanding its activity area [3,4]. West Nile virus (WNV), firstly identified in tropical Africa, has emerged as a cause of outbreaks and sporadic cases in several countries of central, eastern and Mediterranean Europe [5,6].

Little is known about the circulation of these viruses in the southern Balkans, with the exception of Greece and Albania, where high seroprevalence of TOSV [3] and TBEV [4], respectively, has been reported. To increase knowledge about arbovirus circulation in this European sub-region, we conducted a seroprevalence study of infection with phlebovirus TOSV and flaviviruses TBEV and WNV among blood donors in Kosovo.

The study population was represented by blood donors recruited in the Peja Hospital in north-western Kosovo. Of the samples collected from the 301 candidate blood donors screened for donation suitability between 1 January and 30 March 2005, 200 were randomly selected for the study. Informed consent from each participant was obtained. Socio-demographic information included age, gender, occupation, education and area of residence (urban or rural). More detailed information is provided in a previously published study [7].

IgG against TOSV were investigated using an enzyme-linked immunosorbent assay (ELISA) method, which has previously been described [8].

IgG antibodies against TBEV were detected using a commercially available ELISA system (FSME IgG and IgM Immunozym; Progen Biotech GMBH, Heidelberg, Germany). A standard haemagglutination inhibition (HI) test for TBEV and WNV was also performed [9]. HI tests were carried out at pH 6.6, both for TBEV and WNV.

Samples with borderline and positive ELISA results for TOSV were confirmed using a Plaque Reduction Neutralization Test (PRNT) for both TOSV and Sandfly Naples virus (SFNV). Samples with high HI titres for TBEV or WNV were also tested by PRNT [10].

Overall, 200 blood donors were investigated. Of them, 176 (88%) were male and 24 (12%) female. The median age was 34 years (range, 18–62 years). Most participants (i.e. 142, 71.7%) were resident in rural areas, whereas 56 (28.3%)